Anticytotoxic Effect of Green Tea Catechin on *Aggregatibacter actinomycetemcomitans* Vesicles

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**Abstract**

One of the potential virulence factors produced by *Aggregatibacter actinomycetemcomitans* is a leukotoxin, which is secreted to membranous vesicles and to the culture supernatant, generating membrane pores and killing several lymphoid cell types. The lymphoid cells, THP-1 cells exposed to vesicles were lyzed. However, various catechins inhibited the cell lysis by the vesicles. Next, to determine how catchins inhibit vesicles interact with THP-1 cells, SP-DiOC₁₈ labeled vesicles were incubated with catechins and visualized by fluorescens microscopy. Fluorescens microscopy showed that the vesicle did not bind to THP-1 cells in the presence of catechins. These results suggest that the inhibitory mechanisms of catechin on cytotoxic activity could result in the inhibition of binding vesicles to cells by catechins because the catechin component binds to the vesicles.

**Introduction**

*Aggregatibacter actinomycetemcomitans*, a gram negative facultatively anaerobic coccobacillus, is implicated in the etiology of localized aggressive periodontitis (LAP), a severe form of periodontal disease (1, 2), as well as non-oral infections including endocarditis, septicemia, and abscesses (3). *A. actinomycetemcomitans* expresses various adherence factors (4), including fimbriae (5, 6), and invades human epithelial cells (7, 8). *A. actinomycetemcomitans* also produces cytotoxic distending toxin and leukotoxin which target various components of the immune system and may play a role in modulating the host response (9). *A. actinomycetemcomitans* expresses leukotoxin, a member of the RTX (repeats in toxin) family of toxins that kills cells of the lymphocytic and monocytic lineages (10, 11). Outer membrane-derived vesicles secreted by *A. actinomycetemcomitans* contain a membranolytic leukotoxin and the vesicles are toxic to human lymphoid cells (12, 13).

Green tea contains catechins, a class of low molecular weight polyphenols that consist mainly of flavan-3-ol monomers; catechins are present mainly as catechin (C), catechin gallate (Cg), gallocatechin (GC), gallocatechin gallate (GCg), epicatechin (EC), epicatechin gallate (ECg), epigallocatechin (EGC), and epigallocatechin gallate (EGCg). Green tea leaves normally contain 10% to 20% catechins, mainly EGCg (14). A common characteristic of Cg, GCg, ECg and EGCg is the presence of a gallate moiety. The gallate moiety in catechins is responsible for inhibition of enzyme activity (15). Furthermore, it has been reported that gallate-rich compounds like catechins can interact with proteins and lipids (16). In the antimicrobial effect of green tea catechins, pyrogallol catechins (EGCg, EGC, GC and GCg) were more effective than catechol catechins (EC, ECg, C and Cg) against gram-positive bacteria and fungi (17, 18).

In this study, we investigated the antitoxic effect of catechin against *A. actinomycetemcomitans* vesicles and the associated mechanisms.

**Materials and Methods**

**Bacterial strains, cell line and growth conditions**

*A. actinomycetemcomitans* strain ATCC 700685 (JP2 like, highly leukotoxic) was used in the present study. *A. actinomycetemcomitans* was grown in brain heart infusion broth (BHI; Becton, Dickinson and Co., Sparks, MD)
supplemented with 1% Bact™ yeast extract (BHIY; Becton Dickinson) at 37°C for 24 h in an atmosphere of 5% CO₂ in air. Human monocytic THP-1 cells (JCRB0112.1; JCRB Cell Bank, Japan) were maintained in RPMI 1640 (Wako Co., Japan) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), gentamycin (10 μg ml⁻¹) at 37°C in 5% CO₂ in air. Before use, THP-1 cells were washed twice in phosphate buffered saline (PBS; Wako Co., Japan) to remove the medium and suspended in PBS.

**Catechins**

The catechins used in this study were (-)-epigallocatechin gallate (EGCg), (-)-epicatechin gallate (ECg), (+)-catechin (C) and (+)-gallocatechin (GC), and they were purchased from Funakoshi Co. (Tokyo, Japan).

**Purification of A. actinomycetemcomitans vesicles**

Purification of A. actinomycetemcomitans vesicles was performed according to the procedure of Demuth et al. (13). The A. actinomycetemcomitans strain was grown to the late exponential phase in 0.5l of BHIY and harvested by centrifugation at 10,000×g for 10 min, and the culture supernatant was collected. The vesicles were collected by centrifugation at 105,000×g for 30 min. Clear pellets were suspended in PBS, pooled and centrifuged again at 105,000×g. The resulting pellet was suspended in PBS. The protein content of the purified vesicles was determined using a DC protein assay (BioRad, Hercules, CA, USA) using γ-globulin as a standard. The amount of lipopolysaccharide (LPS) of the purified vesicle was determined with a ToxinSensor™ Chromogenic limulus amebocyte lysate (LAL) Endotoxin Assay Kit (Genscript, NJ, USA) according to the manufacturer’s instructions. The endotoxin concentrations were calculated with a standard curve from Escherichia coli endotoxin.

**Influence of green tea catechins on THP-1 cells**

One hundred microliters of reaction mixture, 2×10⁵ cells ml⁻¹ of THP-1 cells, 60 μg protein of purified vesicle ml⁻¹ and 1 mg ml⁻¹ of catechins, were incubated for 60 min at 37°C in an atmosphere of 5% CO₂ in air. The percent cell lysis was calculated for all the reaction mixtures after incubation. The control reaction was without catechins. Next, 1 mg ml⁻¹ of catechins pretreated with THP-1 cells or vesicle was mixed with the intact vesicle or THP-1 cells, respectively, and made up to a final volume of 100μl with PBS. Calculations were performed as described above.

**Influence of catechin on the interaction of A. actinomycetemcomitans vesicles with THP-1 cells**

A. actinomycetemcomitans vesicles were labelled with a lipophilic sulphonated carbocyanine dye, SP-DiOC₁₈ (Molecular Probes, Eugene, OR). Labeling was carried out by mixing 0.1 volumes of 10 μM stock solution of SP-DiOC₁₈ in dimethylsulphoxide with vesicles suspended in PBS and incubating for 30 min at 37°C. The mixture were subsequently transferred to 4°C and incubated for an additional 15 min. Unincorporated dye was removed by centrifuging the vesicles three times at 10,500×g at 4°C for 60 min and washing with PBS. The resulting pellet was resuspended with PBS as SP-DiOC₁₈ labelled vesicles.

The interaction of SP-DiOC₁₈ labelled vesicles with THP-1 cells was carried out by incubating 0.5ml of vesicles (60 μg protein/ml) and 0.5 ml of THP-1 cells (4×10⁶ cells ml⁻¹) with or without 1.0 mg of catechins for 10 min at 37°C. The samples were washed three times with PBS and suspended in 0.2ml PBS. Observations were done using a fluorosccens microscope (BZ-9000, KEYENCE, Osaka, Japan) with a BZ filter GFP-BP (KEYENCE).

**Statistical analysis**

The statistical significance of differences between means was calculated using the two-tailed Student’s t test. A P value of <0.05 was considered significant.

**Results**

**Influence of catechin on THP-1 cells**

The influence of green tea catechins on THP-1 cells was investigated. Catechin untreated THP-1 cells as a control showed 8.0% cell lysis after 60 min incubation. THP-1 cells...
treated with C, ECg, GC and EGCg accounted for 8.0%, 23.0%, 9.7% and 18.0% of cell lysis, respectively (Fig.1A).

Anticytotoxic effect of green tea catechins on A. actinomycetemcomitans vesicles

The total vesicle protein required to lyse 50% of the 2×10⁶ cells of THP-1 cells for 60 min (LD₅₀) was approximately 60 μg for the ATCC 700685 strain (data not shown). The vesicle-mediated lysis of THP-1 cells was inhibited in the presence of 1.0 mg ml⁻¹ green tea catechins, as shown in Fig.1B. The vesicle-mediated lysis in the presence of catechins was observed to be 8.3% by C, 26.0% by ECg, 8.3% by GC and 21.3% by EGCg. Vesicles pretreated with catechin-mediated lysis of THP-1 cells accounted for 9.0% by C, 24.7% by ECg, 16.7% by GC and 26.0% by EGCg (Fig.1C).

On the other hand, vesicle-mediated lysis of THP-1 cells pretreated with catechins accounted for 45.7% by C, 48.7% by ECg, 46.0% by GC and 53.7% by EGCg (Fig.1D).

Furthermore, the influence of LPS in the vesicle was considered. The limulus activity of A. actinomycetemcomitans LPS included in 60 μg protein/ml vesicles was at a very low level. THP-1 cells were not lysed by such A. actinomycetemcomitans LPS levels (data not shown).

Validation of a fluorescence method

Purified A. actinomycetemcomitans vesicles were labelled with a lipophilic sulphonated carbocyanine dye that exhibits negligible spontaneous transfer between intact membranes (13). As shown in Fig. 2A, incubation of SP-DiOC₁₈-labelled vesicles with THP-1 cells resulted in labeling of the cytoplasmic membrane. In contrast, vesicles were scarcely observed at the cell surface in the presence of C (Fig.2B). Also, the results for the presence of GC, ECg and EGCg were similar to the results for the presence of C (data not shown).

Discussion

In the present study, we demonstrated that individual catechin components protect THP-1 cells against death due to A. actinomycetemcomitans vesicles.

As for the influence of green tea catechins on THP-1 cells,
THP-1 cells were slightly lysed by ECg and EGCG. However, C and GC did not influence cell lysis. These results suggest that the gallate moiety slightly induces cell lysis on THP-1 cells.

It has been reported that leukotoxin and LPS are contained in vesicles (12). The level of LPS contained in vesicles had no effect on THP-1 cells (data not shown). Also, it was reported that the vesicle from an isogenic leukotoxin-deficient strain of \textit{A. actinomycetemcomitans} JP2 is non-toxic (13). The vesicle-mediated lysis of THP-1 cells was observed to be 53.0%. It is thought that vesicle-mediated cell lysis is mainly caused by leukotoxin.

The vesicle-mediated lysis of THP-1 cells was strongly inhibited in the presence of green tea catechins, as shown in Fig.1B and C. Furthermore, vesicles were scarcely observed at the cell surface in the presence of green tea catechins. However, the lysis was not inhibited in the catechin pretreated THP-1 cells. Polyphenols have the ability to interact with proteins, lipids and polynucleotides through electrostatic, hydrophobic, and even covalent binding (19, 20, 21, 22). Catechins may have bound to the cell surface, but the vesicles were able to interact with the THP-1 cells pretreated with catechins.

Thus, the cell death was inhibited because catechins bound to the vesicle in advance of the interaction of THP-1 cells and the vesicle.

These results suggest that various catechins interact with vesicles and inhibit combination with THP-1 cells. Furthermore, it is thought that the secretion of leukotoxin from vesicles is inhibited by catechins.

The inhibitory mechanisms of catechin on cytotoxic activity could be a result of binding of individual catechin components to vesicles, which may lead to the aggregation of leukotoxin and the blockage of leukotoxicity.

A cup of tea (about 150ml) contains 250 to 300mg of catechins. Taken together with the bactericidal activity of tea (17, 23), our results further suggest that tea or components of tea may be candidates for prevention of LAP and chronic periodontitis.

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**References**


